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## Raman, IR and CD Spectroscopy on Aggregates and Gels of Lysozyme in Alcohols

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The Structure of lysozyme gels with the protein concentration  $C_p$  of 10 mg/ml formed in 35% alcohol (methyl or ethyl alcohol)+0.075 N HCl has been investigated by Raman and IR spectroscopy. It has been found that the gels maintain a network by forming the antiparallel  $\beta$ -sheet structure as nonlocalized crosslinks. Interaction constants for this  $\beta$ -sheet have been calculated from the Amide I' mode. CD measurements have been made on solutions of the protein in the solvent, 40% EtOH+0.12 N HCl, in the range of  $C_p$  where gelation does not occur. The denatured protein has remained in the monomer state at very low  $C_p$  of 0.01 mg/ml, but aggregated at higher  $C_p$  by forming the  $\beta$ -sheet structure. Fractions of the  $\alpha$ -helix and the  $\beta$ -sheet of the aggregate ( $C_p=4$  mg/ml) have been estimated as  $f_H=0.10$  and  $f_\beta=0.66$ , respectively.

KEY WORDS: Lysozyme/ Gel structure/  $\beta$ -Sheet structure/ Raman spectroscopy/ IR/ CD/ Alcohol/  $\alpha$ -Helix/

### INTRODUCTION

It is known that fibrous proteins form gels quite easily and the gels formed are often important from the biological point of view. For example, fibrin clotting stops a flow of blood by closing a wound.<sup>1)</sup> Globular proteins, on the other hand, transform to gels under very specific conditions. Thermal denaturation at high protein concentrations usually gives turbid aggregates, which do not dissociate to monomers by decreasing temperature. Only a few thermodynamically stable samples which may undergo the reversible sol-gel transformation are known.<sup>1,2)</sup>

In the course of studies on the secondary structure of lysozyme in organic solvents,<sup>3)</sup> we have found that this protein forms gels in 40% acetic acid containing 0.035 N HCl at around 5° with decreasing temperature and returns back to a clear solution by raising temperature. Preliminary tests by using other organic solvents have revealed that similar gelation takes place whenever the protein concentration  $C_p$  is higher than 6 mg/ml and PH is lower than 1.5. It has been also found that transparent gels can be obtained by careful preparation and that they dissociate to monomers at high temperatures. These properties of the lysozyme gels give an opportunity to study not only on the nature of the intermolecular force connecting the molecules but also the gel structure which are still poorly understood.

This paper describes Raman and IR measurements on gels of unlabelled and deuterium-labelled lysozyme and CD measurements on the protein solutions with  $C_p$  less

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than the critical concentration ( $=6$  mg/ml) above which gelation takes place. Methyl and ethyl alcohol have been chosen as organic solvents in the preparation of gels, because these solvents give windows for Raman spectroscopy in frequency regions where informations on the secondary structure of the protein are obtained.

### EXPERIMENTAL

**Materials.** Three times recrystallized hen-egg-white lysozyme (Sigma Chem. Co., Lot No. 57c-8025) was used as test sample without further purification. Ethyl alcohol (EtOH) and hydrochloric acid (HCl) of guaranteed reagent grade were purchased from Nakarai Chemicals, and methyl alcohol (MeOH) of specifically prepared reagent grade and deuterium oxide ( $D_2O$ ) from E. Merck. Deuterium labelled alcohols, MeOD and EtOD, and also DCL were obtained from Commissariat à l'Energie Atomique. Guanidine hydrochloride (GuHCl) was of specifically prepared reagent grade from Nakarai Chemicals. All solvents were used without further purification.

Deuteration of lysozyme was made by the following procedure. The solution of the protein in  $D_2O$  was heated for 2 h at  $50^\circ$  and freeze-dried. The freeze-dried sample was dissolved in fresh  $D_2O$ , heated for one hour at  $50^\circ$  again and then freeze-dried. The samples thus obtained were used as deuterated lysozyme for Raman and IR measurements. GuDCl was obtained from condensation of  $D_2O$  solution of GuHCl by heating gently.

Table I gives the protein concentrations and the solvent compositions of the solutions and gels used for respective measurements. The protein concentrations of the samples Cl-3 were estimated with a spectrophotometer (Curl Zeiss, Model PMQ II) by the use of the extinction coefficient,  $E_{280nm}^{1\%} = 26.35$ . For Raman and IR measurements, the solutions (R1, R2, R7, R8) were prepared by dissolving weighed amount of the protein in the solvents. For the preparation of the gels (R3-6), a solution of the protein in pure  $H_2O$  and an aqueous solution of alcohol and HCl were prepared separately. The concentrations were adjusted

Table I

Method	Sample code	Lysozyme Conc. (mg/ml)	Solvent	Structure
Raman	R1	100	$H_2O$	Native <sup>b</sup>
Scattering	R2	100	$D_2O$	Native <sup>b</sup>
	R3	100	40% MeOH, 0.075 N HCl, $H_2O$	Denatured, Gel <sup>a</sup>
	R4	100	40% MeOD, 0.075 N DCl, $D_2O$	Denatured, Gel <sup>a</sup>
	R5	100	35% EtOH, 0.075 N HCl, $H_2O$	Denatured, Gel <sup>a</sup>
	R6	100	35% EtOD, 0.075 N DCl, $D_2O$	Denatured, Gel <sup>a</sup>
	R7	100	6 M GuHCl, $H_2O$	Random Coil <sup>b</sup>
	R8	100	6 M GuDCl, $D_2O$	Random Coil <sup>b</sup>
Infrared	R2	100	$D_2O$	Native <sup>b</sup>
Absorption	R6	100	35% EtOD, 0.075 N DCl, $D_2O$	Denatured, Gel <sup>a</sup>
Circular	C1	0.01	40% EtOH, 0.12 N HCl, $H_2O$	Denatured <sup>a</sup>
Dichronism	C2	1.0	40% EtOH, 0.12 N HCl, $H_2O$	Denatured <sup>a</sup>
	C3	4.0	40% EtOH, 0.12 N HCl, $H_2O$	Denatured <sup>a</sup>

a. This work.    b. Reference 3.

to give prescribed values in the Table I after mixing them by the volume ratio of 1 : 1. Mixing was done by rapid stirring. Only this procedure provided reproducible transparent gels.

**Methods.** Raman scattering measurements were made with a laser Raman spectrometer recently constructed in our laboratory. A 6 W argon ion laser with an oven stabilized etalon was used as light source. The incident beam ( $\lambda=488$  nm) was focused by a lens into a cylindrical cell (i.d.: 2 mm) from the bottom. Grating of the light scattered from the samples was made with a Jobin-Yvon THR1500 monochrometer. The intensity was recorded as digitized data to facilitate data processing with a microcomputer (Union Giken, System 77). In order to reduce stray light due to Rayleigh scattering, especially intense for gels, color filters which cut light with wavelength shorter than 500 nm was inserted between the entrance slit and the lens. Therefore reliable Raman spectra were obtained at frequencies above  $700\text{ cm}^{-1}$ . Measurements were made at  $25.0\pm0.1^\circ$ . Before measurements, all samples were irradiated for about 30 minutes to reduce fluorescence.

IR measurements were made with a Perkin-Elmer 521 doublebeam spectrophotometer by using a demountable cell with KRS-5 windows and platinum spacers with a thickness of 0.2 mm. The reference cell was filled with solvents which have the same composition as those of either the solutions or gels. All measurements were made at room temperatures.

CD measurements were made with a Duram-Jasco J-20 spectropolarimeter. The cell was put under nitrogen atmosphere and the temperature was controlled by circulating water around the cell from a thermostatted water circuit. The data were expressed in terms of the mean residue ellipticity  $[\theta]$  by taking the mean residue weight of the protein as 111, which was calculated from its amino acid composition.

## RESULTS

**Raman Spectra.** Laser Raman spectroscopic studies on aqueous solutions of lysozyme in the native or denatured state have been made by Lord et al.<sup>4-7)</sup> and also by several other investigators.<sup>8-10)</sup> The spectrum of the 10% aqueous solution of lysozyme in the native state (the sample R1) obtained by our instrument agreed quite well with the data by Lord and Yu<sup>4)</sup> over the frequency region from 700 to  $1800\text{ cm}^{-1}$  not only in frequency to an uncertainty of  $2\text{ cm}^{-1}$  but also in relative intensity.

The conformation of the protein backbone in which we are interested can be effectively deduced from the Amide III ( $1200\text{--}1300\text{ cm}^{-1}$ ) and the Amide I ( $1620\text{--}1720\text{ cm}^{-1}$ ) modes. Concerning the Amide III, the spectrum of the gel prepared by using MeOH (the sample R3) has been compared with those of the samples R1 and R7 for which the protein is known to have the native and the random coiled conformation, respectively.<sup>9)</sup> In Figure 1, the spectra of the above three samples are shown in the region of  $1170\text{--}1390\text{ cm}^{-1}$ . According to studies on the relation between Raman lines of the Amide III mode and the secondary structure of synthetic polypeptides,<sup>11-14)</sup> the Amide III mode appears at frequencies from  $1265$  to  $1295\text{ cm}^{-1}$  for the  $\alpha$ -helix, at  $1245\pm3\text{ cm}^{-1}$  for the random form, and at  $1235\pm5\text{ cm}^{-1}$  for the antiparallel  $\beta$ -sheet structure. On the basis of this criterion, the intense

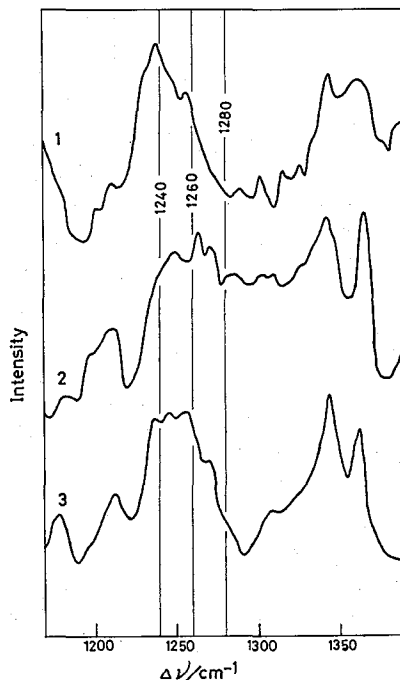


Fig. 1. Raman spectra in the Amide III region. Curve 1, gel in 40% MeOH+0.075 N HCl; 2, native in aqueous solution; 3, random coil in 6 M GuHCl.

peak of the gel (curve 1) at  $1238\text{ cm}^{-1}$  may be related to the antiparallel  $\beta$ -sheet. The decrease in intensity in the region  $1260\text{--}1280\text{ cm}^{-1}$  for the curve 1 as compared with the spectrum of the native protein (curve 2) is considered to reflect the decrease in the  $\alpha$ -helical content. This conjecture is supported by facts that the Raman line at  $933\text{ cm}^{-1}$  characteristic of the  $\alpha$ -helix<sup>11)</sup> has been observed as a sharp peak for the native protein, but disappeared for the both gels prepared by using MeOH and EtOH. The Amide III in the random coiled state (curve 3) are broad and looks to have several small peaks. This implies that two rotational angles around  $\text{C}^\alpha\text{-C}$  and  $\text{N-C}^\alpha$  bonds may take various values which lead to the random coiled conformation. Aside from the Amide III mode, two lines have newly appeared at  $1303$  and  $1316\text{ cm}^{-1}$  for the gel. These lines might be related to the  $\beta$ -turn when we adopt the calculation by Krim and Bandekar.<sup>15)</sup>

No useful information on the Amide III mode could be derived from the spectrum of the gel R5, because ethyl alcohol used as the organic solvent for the preparation of the gel has an intense Raman line around  $1280\text{ cm}^{-1}$  which masks a large portion of the Amide III mode.

Figure 2 shows the spectra of the two gels (R4, R6) and the two solutions (R2, R8) in the frequency region of  $1500\text{--}1750\text{ cm}^{-1}$  where the Amide I (or I' for deuterium labelled samples) is observed. Here all materials were substituted for their deuterium labelled compounds in order to avoid ambiguity arising from the overlapping of the broad band due to  $\text{H}_2\text{O}$ . According to studies on the Amide I mode of synthetic polypeptides,<sup>11)</sup> a Raman line appears at  $1645\text{--}1657\text{ cm}^{-1}$  for the  $\alpha$ -helix, and at  $1665\text{--}1672\text{ cm}^{-1}$  for the

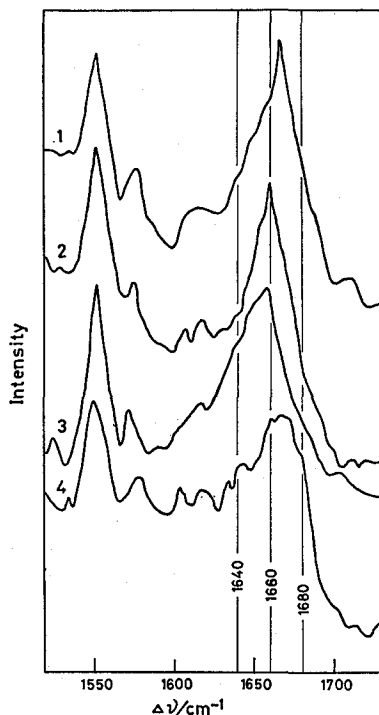


Fig. 2. Raman spectra in the Amide I' region. Curve 1, gel in 35% EtOD+0.075 N DCl; 2, gel in 40% MeOD+0.075 N DCl; 3, native in D<sub>2</sub>O solution; 4, random coil in 6 M GuDCl.

$\beta$ -sheet. For the random coiled structure it appears at about the same frequency range as for the  $\beta$ -sheet, but its bandwidth becomes much broader. The native protein (curve 3) has a peak at  $1658\text{ cm}^{-1}$  which is broadened asymmetrically to the low frequency side where the Raman line for the  $\alpha$ -helix should be seen. Taking into account that the Amide I line has appeared at  $1671\text{ cm}^{-1}$  for the undeuterated gels and that the deuteration of the N-H in peptide groups might induce a decrease of 5 to  $10\text{ cm}^{-1}$  in the Amide I frequency,<sup>11)</sup> intense lines at  $1665$  and  $1660\text{ cm}^{-1}$  observed for the gels in Fig. 2 may be assigned to the  $\beta$ -sheet. This assignment is consistent with the results of the Amide III mode. The sharpness of the Amide I' suggests that conformations of the molecules taking the  $\beta$ -sheet structure are more or less alike.

*IR Spectra.* Since the Amide I is also IR active, infrared measurements have been made on the two deuterated samples, R2 and R6 in the range of frequency from  $1400$  to  $2000\text{ cm}^{-1}$ . The results are shown in Fig. 3. The spectrum of the native protein in D<sub>2</sub>O (curve 1) has an absorption peak at  $1650\text{ cm}^{-1}$  due to the  $\alpha$ -helical or random structure, and a shoulder at  $1630\text{ cm}^{-1}$  due to the intramolecular antiparallel  $\beta$ -sheet.<sup>33)</sup> The spectrum of the gel (curve 2) exhibits a new peak at  $1620\text{ cm}^{-1}$  and two shoulders at  $1960$  and  $1715\text{ cm}^{-1}$ . The absorption bands at  $1620$  and  $1690\text{ cm}^{-1}$  have been observed for silk fibroin which takes the intermolecular antiparallel  $\beta$ -sheet structure.<sup>16)</sup> Two shoulders at  $1620$  and  $1685\text{ cm}^{-1}$  have been also observed for solutions of  $\beta$ -lactoglobulin

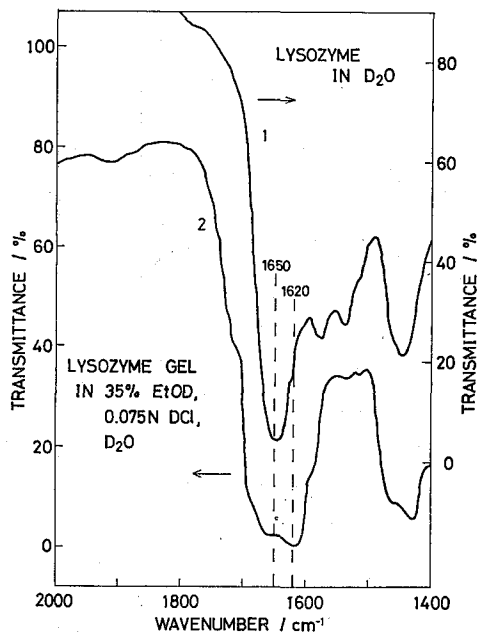


Fig. 3. IR spectra in the Amide I' region. Curve 1, native in  $D_2O$  solution; 2, gel in 35% EtOD+0.075 N DCl.

in 40% MeOD taking the  $\beta$ -sheet structure due to aggregation.<sup>17)</sup> Thus, results of IR measurements give an additional support for that a large fraction of the  $\beta$ -sheet is characteristic of the secondary structure of the gel.

**CD Spectra.** In order to examine the protein structure in dilute concentration range where gelation does not take place, CD measurements have been carried out on lysozyme dissolved in the solvent, 40% EtOH+0.12 N HCl, and  $[\theta]$  has been studied as a function of time and concentration. This solvent composition is slightly different from that used for Raman measurements. However it has been found that the effect of this difference on  $[\theta]$  is small. Figure 4 shows the time dependence of  $[\theta]$  for the sample C2 ( $C_p = 1$  mg/ml) at 25°. Here the dotted line represents  $[\theta]$  of the native protein in  $H_2O$ . The spectra in the long wavelength region show that the protein has become quickly denatured in this solvent system (curve 1) and also that the environment of side-chain residues has changed gradually with time (curve 2-4). In the short wavelength region, the value of  $-[\theta]$  at 208 nm has increased at the very initial stage after the preparation of the sample, and then decreased gradually as time elapsed. Such an increase in  $-[\theta]_{208}$  has been observed for lysozyme denatured in 30-60% propanol without HCl and has been interpreted to reflect the conformational change of the molecule to the  $\alpha$ -helix rich conformation.<sup>3)</sup> The decrease in  $-[\theta]_{208}$  has been accompanied with the gradual change of the whole spectrum in shape in such a way that  $[\theta]$  finally has given one minimum at 216 nm. CD curves similar to the curve 4 have been derived for poly L-lysine<sup>18-20)</sup> and also for silk fibroin.<sup>16)</sup> Both of them have the antiparallel  $\beta$ -sheet structure. Therefore the time dependence shown in Figure 4 can be interpreted to exhibit the process that the denatured

# Structure of Lysozyme Gels in Alcohols

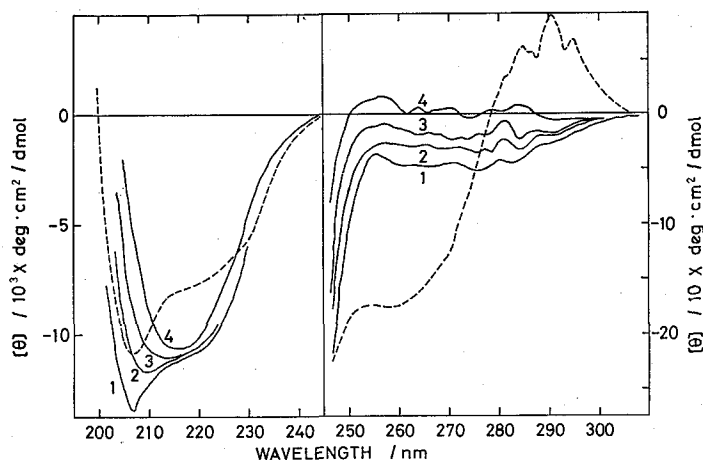


Fig. 4. Time dependence of  $[\theta]$  of lysozyme in 40% EtOH+0.12 N HCl ( $C_p=1$  mg/ml). Measurements were carried out  $t$  hrs later after preparation of samples; curve 1,  $t=0$ ; 2,  $t=2$  h; 3,  $t=6$  h; 4,  $t=1$  week. Dotted lines represent data of lysozyme in aqueous solution.

lysozyme converts from the  $\alpha$ -helix rich conformation to the  $\beta$ -sheet rich conformation.

The concentration dependence of  $[\theta]$  has been studied for three solutions all kept standing for 12 hrs after the preparation of the samples. The results shown in Fig. 5 indicate that the  $\beta$ -sheet structure found for the sample C2 is not intramolecular but intermolecular one. At the protein concentration as low as 0.01 mg/ml, the protein remains as the monomer with the  $\alpha$ -helix rich conformation, but at  $C_p=1.0$  and 4.0 mg/ml the  $\beta$ -sheet structure becomes dominant. Another support for the intermolecular nature of the  $\beta$ -sheet structure is that the conversion from the  $\alpha$ -helix to the  $\beta$ -sheet has been accelerated by increasing the protein concentration. Experiments on the temperature

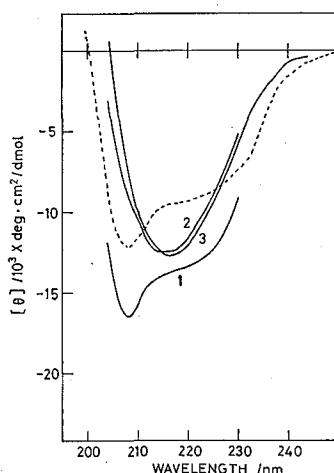


Fig. 5. Concentration dependence of  $[\theta]$  of lysozyme in 40% EtOH+0.12 N HCl. Curve 1,  $C_p=0.01$ ; 2,  $C_p=1$ ; 3,  $C_p=4$  (mg/ml). The dotted line represents  $[\theta]$  of lysozyme in aqueous solution.



dependence of  $[\theta]$  on the sample C2 have also shown that at temperatures above 45°, the spectrum has become characteristic of the monomer and returned to the same spectrum as the curve 4 in Fig. 4 when temperature is lowered to 25°. Thus we may conclude that in this solvent system, the formation of hydrogen bonding between different molecules stabilizes denatured lysozyme at concentration as low as 1 mg/ml, and that the intermolecular  $\beta$ -sheet structure acts as nonlocalized crosslinks for formation of the gel network at  $C_p$  higher than 6 mg/ml.

## DISCUSSION

Studies on gels of globular proteins are few in literatures. Jirgensons<sup>21)</sup> has reported that a 2% solution of egg albumin in 40-60% n-propyl alcohol formed a gel which liquefied reversibly above 67°. Any spectroscopic measurement has not been made to study the gel structure. Lin and Yu<sup>22)</sup> have found that glucagon with 75%  $\alpha$ -helix content has denatured in acidic solutions to a random coil, and formed a gel on standing at 26°. Together with the transition from the  $\alpha$ -helix to the gel, the Amide III frequency has shifted from 1266 to 1232  $\text{cm}^{-1}$  and an intense Amide I line has appeared at 1672  $\text{cm}^{-1}$  in the Raman spectrum. Based on these observations, they have concluded that the gel has the  $\beta$ -sheet structure. It has been already described that silk fibroin, a typical fibrous protein, can form a stable gel by taking the intermolecular  $\beta$ -sheet structure. These works and the present results strongly suggest that, in spite of differences in native conformation and biological function between globular and fibrous proteins, the formation and stabilization of their gel networks are achieved by the same type of the force as the hydrogen bonding between different molecules.

Since the highest concentration,  $C_p=4$  mg/ml, of the solution used for CD measurements is close to the critical concentration for gelation to occur ( $C_p^*=6$  mg/ml) and also the two CD curves 2 and 3 of  $C_p=1$  and 4 mg/ml in Fig. 5 are almost coincident, we might assume that the secondary structure of the gel is roughly the same as that of the solution with  $C_p=4$  mg/ml. Therefore we have tried to estimate fractions of the  $\alpha$ -helix, the  $\beta$ -sheet and the random form,  $f_i$  ( $i=H, \beta, R$ ), from the analysis of the curve 3 in Fig. 5 following the method developed by Greenfield and Fasman.<sup>24)</sup> According to their method,  $[\theta]_\lambda$ , an experimental value at wavelength  $\lambda$ , can be expressed by Eq. (1).

$$[\theta]_\lambda = f_H[\theta]_\lambda^H + f_\beta[\theta]_\lambda^\beta + f_R[\theta]_\lambda^R \quad (1)$$

where  $[\theta]_\lambda^H$ ,  $[\theta]_\lambda^\beta$  and  $[\theta]_\lambda^R$  are reference values for the pure  $\alpha$ -helix,  $\beta$ -sheet and random form.  $f_H$ ,  $f_\beta$  and  $f_R$  are determined with a least square method under conditions,  $f_H + f_\beta + f_R = 1$  and all  $f_i \geq 0$ . When CD data of poly L-lysine have been used as reference values,<sup>24)</sup> we have obtained  $f_H=0.10$ ,  $f_\beta=0.66$  and consequently  $f_R=0.24$ . As Fig. 6 shows,  $[\theta]_\lambda$  values calculated from Eq. (1) with the above  $f$  values fall closely on the experimental curve. The maximum deviation is less than 4% and the standard deviation (STD) is 49  $\text{deg cm}^2/\text{dmol}$ . The secondary structure of the native lysozyme has been reported as  $f_H=0.29-0.42$ ,  $f_\beta=0.10$  and  $f_R=0.62-0.48$  from X-ray diffraction studies.<sup>25,26)</sup> Therefore the aggregation process is characterized by a large increase in the  $\beta$ -sheet content accompanied by a decrease in the  $\alpha$ -helix content. This is consistent with the

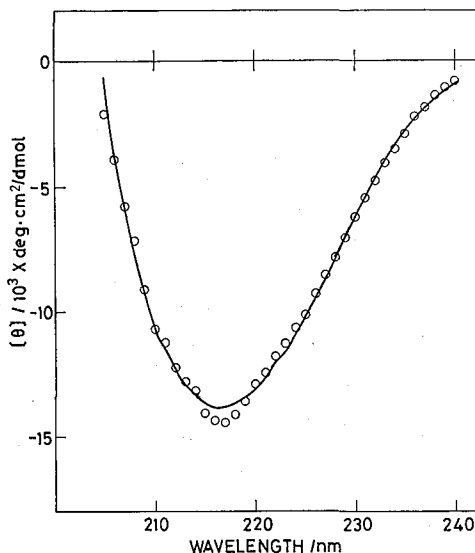


Fig. 6. Comparison between calculated and experimental CD curves. Open circles are calculated from Eq. (1) with  $f_H=0.10$  and  $f_\beta=0.66$ , and  $[\theta]_{\lambda^i}$  ( $i=H, \beta, R$ ) of PLL data. Full line is the CD curve 3 in Fig. 5.

results of both Raman and IR measurements on the lysozyme gels. The large value of  $f_\beta=0.66$  suggests that the chains in the aggregates are more or less extended.

As reference values for  $[\theta]_{\lambda^i}$  ( $i=H, \beta, R$ ), we may also use values computed from CD spectra of five proteins with the  $f$  values obtained by X-ray diffraction studies.<sup>27,28)</sup> The analysis has given  $f_H=0.27$ ,  $f_\beta=0.23$  and  $f_R=0.50$ . Fitting with these  $f$  values by Eq. (1), however, has been very poor to the extent that the STD has become as large as 420. This poor fitting may arise from a fact that the  $\beta$  structure of the proteins used for the computation of the reference values are formed by intramolecular hydrogen bonding, whereas  $[\theta]_{\lambda^\beta}$  of PLL represents the behavior of the intermolecular  $\beta$  structure.

It is still ambiguous whether the use of  $[\theta]$  of the synthetic polypeptide is permissible for the calculation of  $f$  values of globular proteins. Thus  $f$  values estimated by using PLL data might be subject to considerable uncertainty. Nevertheless, fitting by using several combinations of the PLL data and the computed CD data as  $[\theta]_{\lambda^i}$  has revealed that good agreement between calculated and experimental values has been obtained only if  $[\theta]_{\lambda^\beta}$  of PLL has been used. For example, the use of the PLL data for  $[\theta]_{\lambda^H}$  and  $[\theta]_{\lambda^\beta}$  and of the computed data for  $[\theta]_{\lambda^R}$  has given  $f_H=0.09$ ,  $f_\beta=0.56$  and  $STD=42$ . This indicates that we must be careful of the choice of reference values when Eq. (1) is applied for the analysis of CD data.

The presence of a large amount of the  $\beta$  structure in the gels permits us to evaluate interaction parameters between intra and inter-molecular peptide groups from the Amide I' mode. The data of the gel formed in EtOD shown in Figs. 2 and 3 have been analysed with the method developed first by Miyazawa<sup>29)</sup> and refined later by other investigators.<sup>30,31)</sup> The Amide I' mode has four characteristic frequencies  $\nu(\delta, \delta')$  where  $\delta$  and  $\delta'$  represent the phase difference between intra- and inter- molecular carbonyl residues, respectively

and take either 0 or  $\pi$ .  $\nu(\delta, \delta')$  can be written by using intermolecular interaction constants  $D_{01}$  and  $D_{11}$  and intramolecular one  $D_{10}$  as Eqs. (2)–(5).

$$\nu(0, 0) = \nu_0 + D_{10} + D_{01} + D_{11} \quad (2)$$

$$\nu(\pi, 0) = \nu_0 - D_{10} + D_{01} + D_{11} \quad (3)$$

$$\nu(0, \pi) = \nu_0 + D_{10} - D_{01} - D_{11} \quad (4)$$

$$\nu(\pi, \pi) = \nu_0 - D_{10} - D_{01} + D_{11} \quad (5)$$

Here  $\nu_0$  is the unperturbed frequency. The Raman active and IR inactive  $\nu(0, 0)$  is taken as  $1665 \text{ cm}^{-1}$  from the curve 1 in Fig. 2, and  $\nu(\pi, 0)$  and  $\nu(0, \pi)$  may be assigned as  $1620$  and  $1690 \text{ cm}^{-1}$  from Fig. 3, respectively. The  $\nu(\pi, \pi)$  mode is both Raman and IR active but its intensity is believed too weak to be observed. For this gel, we noticed a shoulder at  $1715 \text{ cm}^{-1}$  in the IR spectrum and a Raman line at  $1714 \text{ cm}^{-1}$ . Thus we tentatively assigns  $\nu(\pi, \pi) = 1715 \text{ cm}^{-1}$ . Substitution of the above values into  $\nu(\delta, \delta')$  of Eqs. (2)–(5) has given  $\nu_0 = 1672.5$ ,  $D_{10} = -5$ ,  $D_{01} = -30$  and  $D_{11} = 17.5 \text{ cm}^{-1}$ . At present there is no systematic study on the Amide I' splitting of deuterated crystalline polypeptides, and the effect of deuteration on the Amide I mode is not clarified theoretically, even though it is supposed to be small.<sup>33)</sup> Here we give a few comments from comparison of  $D$  values of the deuterated gel with those of undeuterated crystalline polypeptides ([Ala]<sub>n</sub>, [Ala-Gly]<sub>n</sub>, [Gly I]<sub>n</sub>) in Table V of the reference 32. First, signs of interaction constants are all in agreement with those for polypeptides. This indicates that a similar transition dipole coupling mechanism as suggested by Krimm and Abe<sup>30)</sup> is responsible for the Amide I' splitting of the gel. Second,  $D_{10}$  of the gel is smaller than  $D_{10}$  of polypeptides,  $8 \sim 11$ , while the absolute magnitudes of  $D_{01}$  and  $D_{11}$  are both larger than corresponding values of polypeptides,  $D_{01} = -16 \sim -26$  and  $D_{11} = 7 \sim 11$ . If the effect of deuteration on  $D$  could be assumed to be small, the intermolecular interaction seems to play a more important role for stabilization of the gel in comparison with the case for the crystalline structure of polypeptides.

Finally we may picture the lysozyme gel as follows; a major portion of more or less extended chains are densely packed at crosslink loci, of at least two per chain, due to the formations of the  $\beta$ -sheet structure, and the remaining portion, taking the random form, is sparsely distributed in solvents as effective network strands.

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# Structure of Lysozyme Gels in Alcohols

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